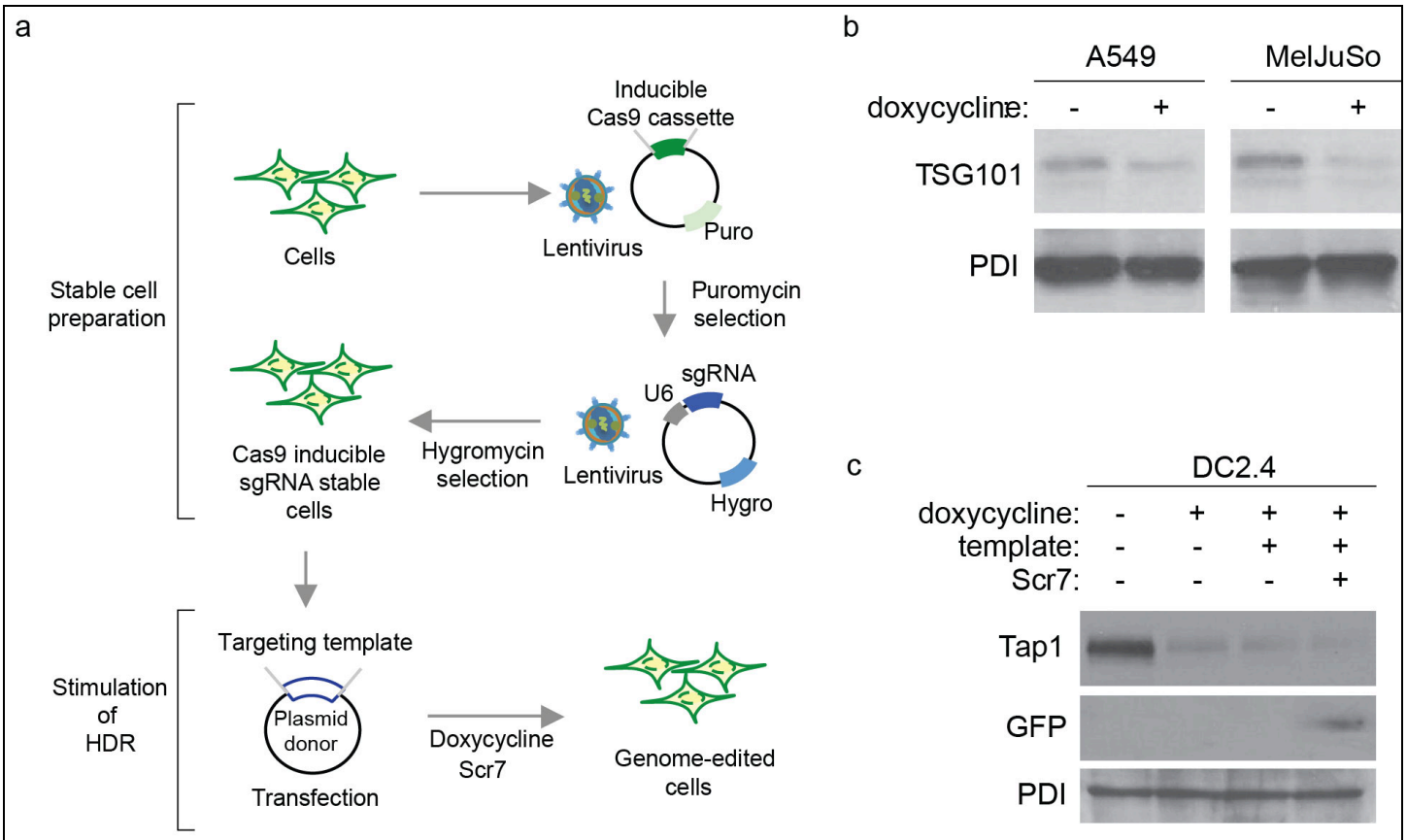
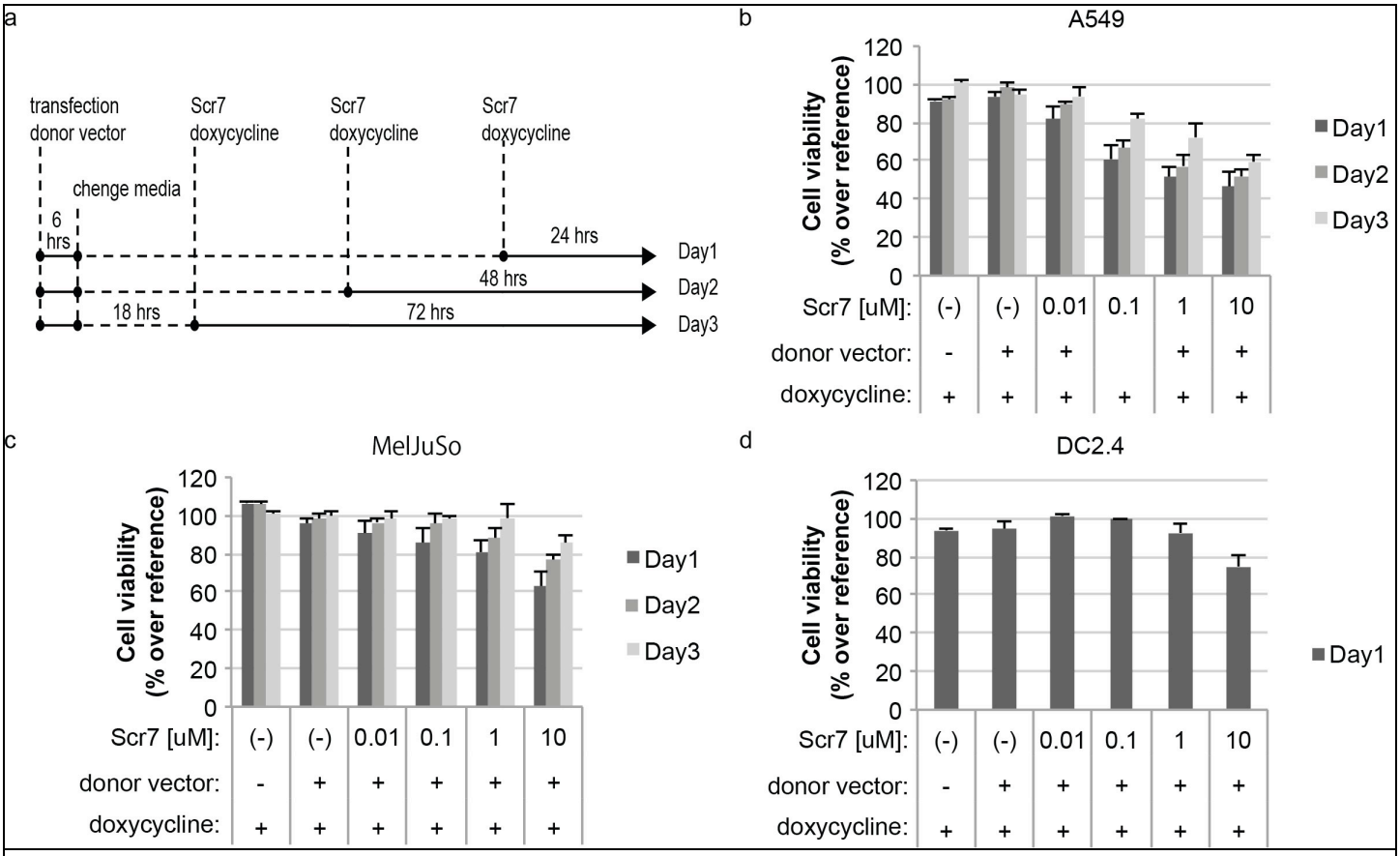


Supplementary Figure 1

Knockout efficiency of TSG101 and Tap1 protein

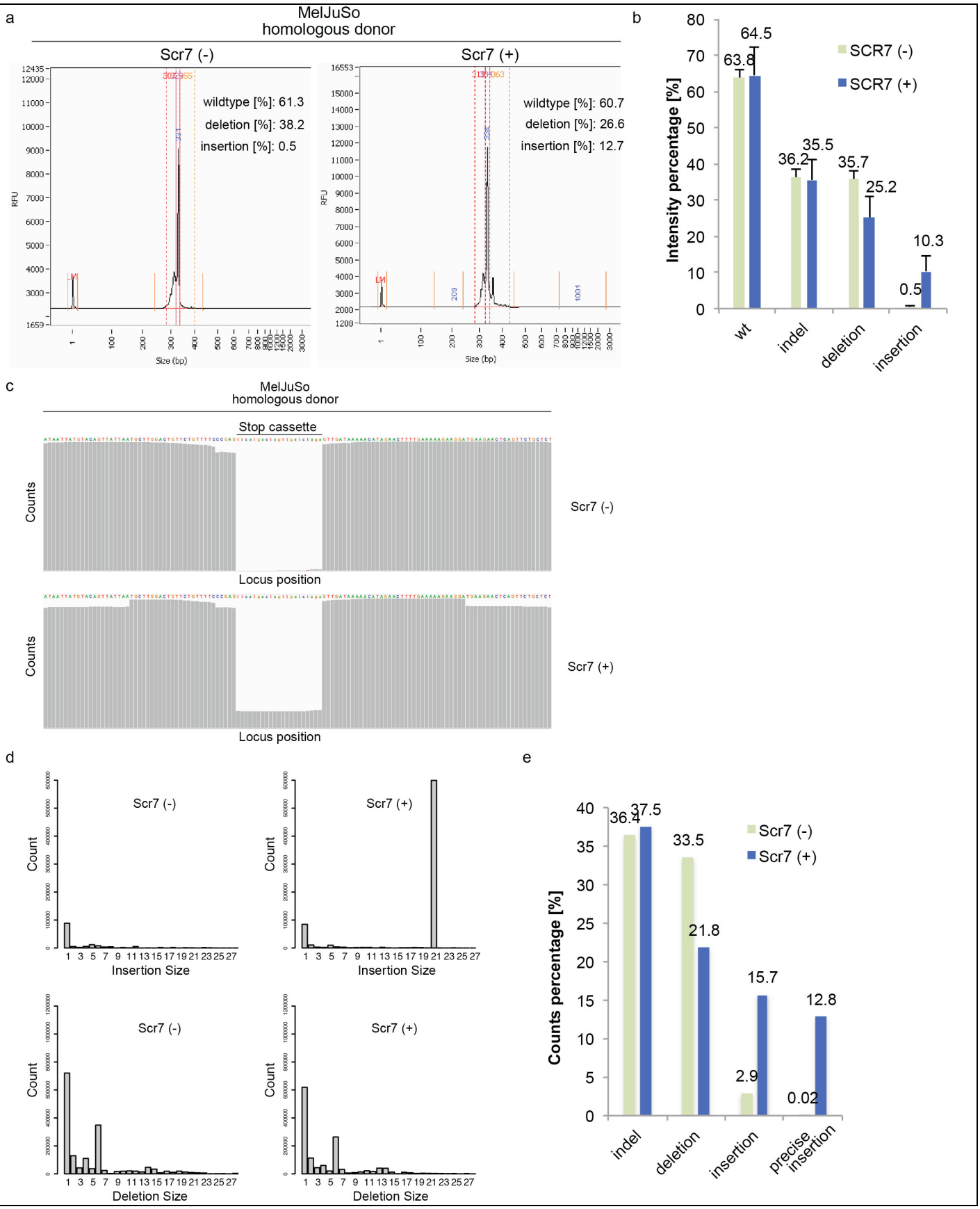
(a) Schematic representation of the experimental setup: Cells were transduced with lentivirus to install a doxycycline-inducible Cas9 cassette and the puromycin resistance gene; Cas9-inducible cells were selected in puromycin-containing medium. The resulting cells were then transduced using a lentivirus vector carrying U6 promotor- single guide (sg) RNA and the hygromycin resistance gene, and underwent hygromycin selection. The Cas9-inducible cells stably expressing sgRNA were transfected with a plasmid donor, to enable homology directed repair (HDR) upon Cas9 induction by addition of doxycycline in the presence or absence of Scr7. (b) and (c). The Cas9-inducible A549 and MelJuSo cells stably expressing *TSG101* sgRNA (b), or the Cas9-inducible DC2.4 cells stably expressing *Tap1* sgRNA (c) were treated with 1 μg/mL doxycycline. After 72 hours of treatment, cell lysates from these mixed populations were analyzed by SDS-PAGE. The TSG101 protein, the Tap1 protein, and Venus protein were detected by immunoblot using anti-TSG101, anti-Tap1 and GFP antibody, respectively.



Supplementary Figure 2

Cell viability upon treatment with Scr7

(a) Schematic representation of the MTT assay. Cells were transfected with the plasmid donor. After 6 hours, the cells were cultured in fresh media for 18 hours. At the indicated time point (24 hrs to 72 hrs) prior to the MTT assay, cells were treated with both Scr7 and doxycycline. Cell viability was then measured by the MTT assay. Absorbance at 550 nm was normalized to that of reference wells; cell viability is shown as a percentage increase over the reference. (n = 3, mean ± SD)

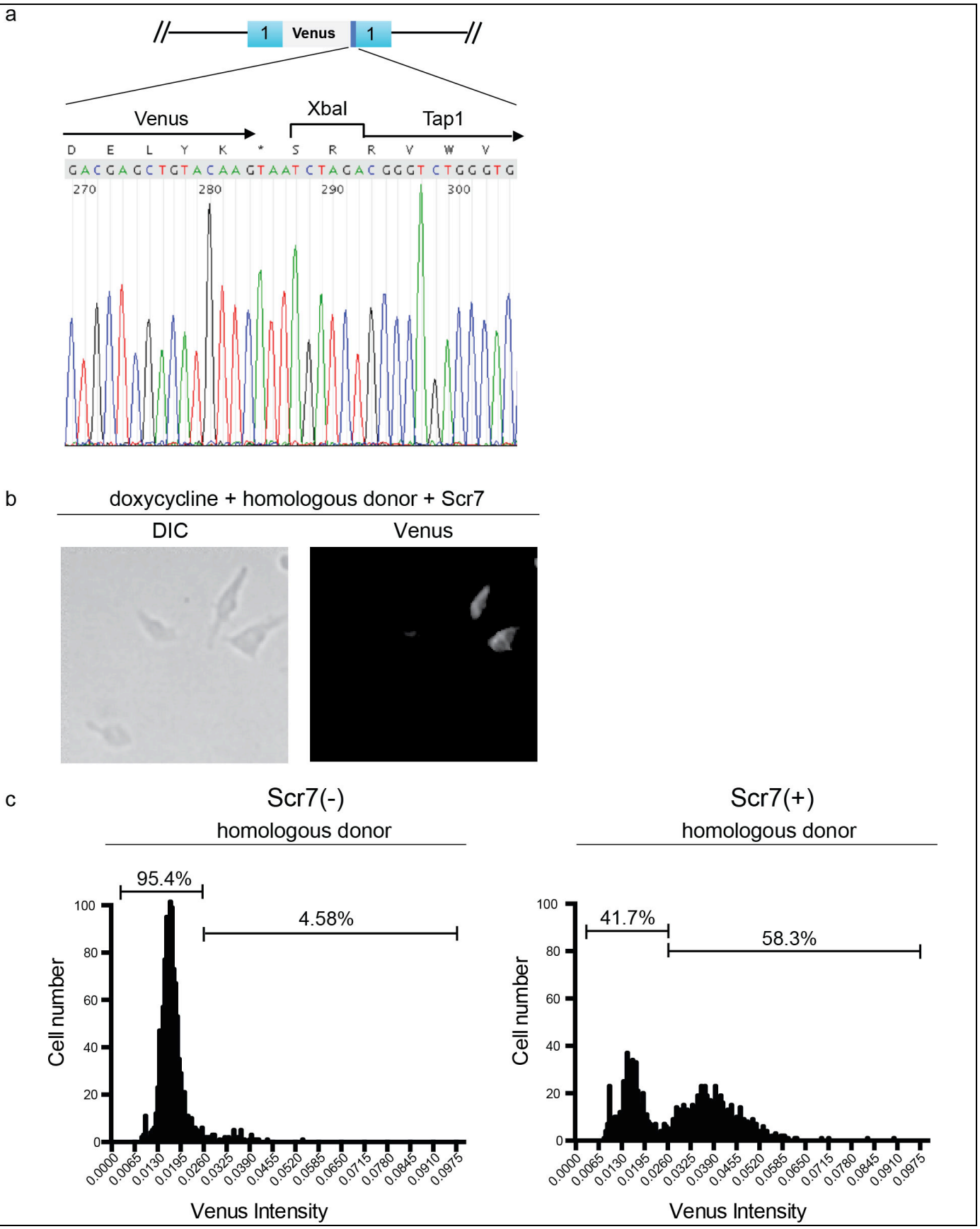

a
MelJuSo
homologous donor
Scr7 (-)
Scr7 (+)
wildtype [%]: 61.3
deletion [%]: 38.2
insertion [%]: 0.5
wildtype [%]: 60.7
deletion [%]: 26.6
insertion [%]: 12.7
RFU
Size (bp)
b
Intensity percentage [%]
SCR7 (-)
SCR7 (+)
63.8
64.5
36.2
35.5
35.7
25.2
0.5
10.3
wt
indel
deletion
insertion
c
MelJuSo
homologous donor
Stop cassette
Counts
Scr7 (-)
Scr7 (+)
Locus position
d
Scr7 (-)
Scr7 (+)
Count
Insertion Size
Deletion Size
e
Counts percentage [%]
Scr7 (-)
Scr7 (+)
36.4
37.5
33.5
21.8
2.9
15.7
0.02
12.8
indel
deletion
insertion
precise insertion

Supplementary Figure 3

Quantification of indel frequency by capillary electrophoresis and deep sequencing

(a and b) Quantification of indel frequency by chip-based capillary electrophoresis. (a) A 318 bp segment in the proximity of the PAM motif was amplified from MelJuSo cells in Fig. 1d by PCR as described in the Methods section. PCR products were run on a chip-based capillary electrophoresis analyzer. The main peaks are the wild-type amplicons (mobility: 318 bp) of the *TSG101* locus in proximity to the sgRNA targeting site. Deletion fragments were observed on the left side of the main peak. ~20 bp larger amplicons were observed to the right of the main peak-- evidence of the stop cassette-insertion. The data shown is representative of 3 individual experiments. (b) Average frequencies are shown (n = 3, mean ± s.d.)

(c-e) Quantification of indel frequency by deep sequencing. (c) Count frequency at each base in the stop cassette-inserted *TSG101* locus is shown as a histogram. (d) Insertion and deletion patterns observed are shown as histograms. A 21 bp precise insertion was observed at the right upper panel— evidence of a precise insertion. (e) The percentage of indel-, deletion-, insertion- and stop cassette-precise insertion-counts relative to total counts in each condition are shown as a graph.

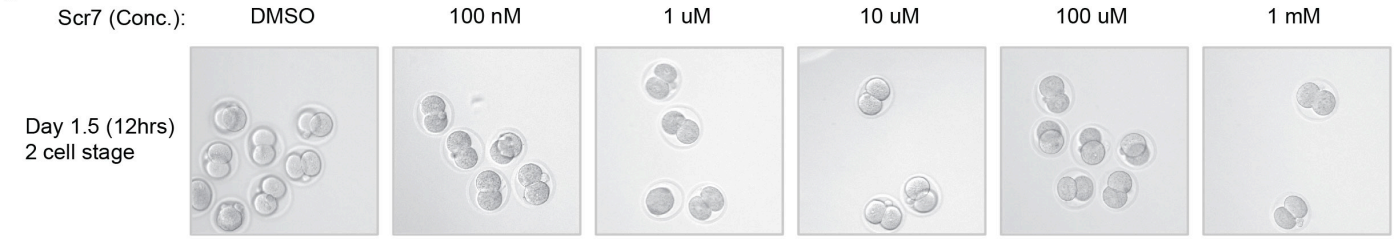

a
1
Venus
1
Venus
XbaI
Tap1
D E L Y K * S R R V W V
GACGAGCTGTACAAGTAATCTAGACGGGTCTGGGTG
270
280
290
300
b
doxycycline + homologous donor + Scr7
DIC
Venus
c
Scr7(-)
homologous donor
Scr7(+)
homologous donor
95.4%
4.58%
41.7%
58.3%
Cell number
Venus Intensity
0
20
40
60
80
100
0.0000
0.0065
0.0130
0.0195
0.0260
0.0325
0.0390
0.0455
0.0520
0.0585
0.0650
0.0715
0.0780
0.0845
0.0910
0.0975

Supplementary Figure 4

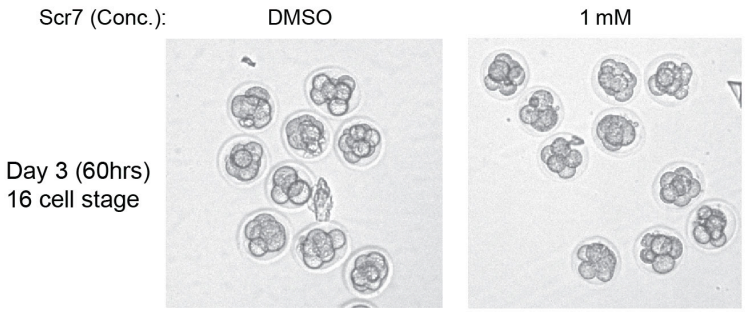
Representative sequencing result, microscopy images and imaging analysis of the targeted cells at the Tap1 locus

(a) The bands indicated by an arrow in Fig. 1h were gel-purified and analyzed by sequencing. The XbaI restriction site that was introduced by the template DNA construction was present at the 3' end of the Venus gene stop codon. (b and c) Images of the cells treated with or without Scr7 in Fig. 1g were acquired by fluorescence microscopy (b: images of DIC channel and GFP channel of the donor vector-transfected cells treated with Scr7), and the images were analyzed by CellProfiler (see the supplementary method). Fluorescence intensities are presented as a histogram in (c)-- horizontal axis: fluorescence intensity, vertical axis: cell number in each bin.

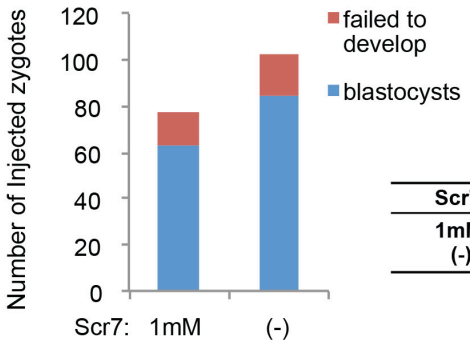
a



b



c

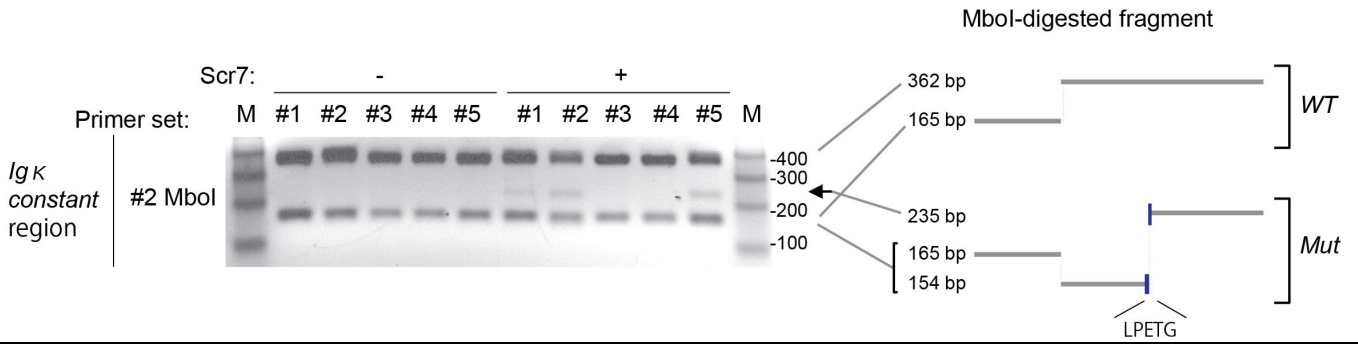


Scr7	blastocyst	failed to develop	total
1mM	63	15	78
(-)	84	19	103

d

```
5’ CCTCTGCCCGCTGCAAGCTCTGGTAAAACTTGGCTACCAAAGAGACTGATGTAAATGCAT  wt
   CCTCTGCCCGCTGCAA----------CTTGGCTACCAAAGAGACTGATGTAAATGCAT  -12
   CCTCTGCCCGCTGCAAGCTC---------TTGGCTACCAAAGAGACTGATGTAAATGCAT  -8
   CCTCTGCCCGCTGCAAGCTCTGGTAA-----GGCTACCAAAGAGACTGATGTAAATGCAT  -5
   CCTCTGCCCGCTGCAAGCTCTGGTAAAACTT--CTACCAAAGAGACTGATGTAAATGCAT  -2
   CCTCTGCCCGCTGCAAGCTCTGGTAAAACTTG-CTACCAAAGAGACTGATGTAAATGCAT  -1
   CCTCTGCCCGCTGCAAGCTCTGGTAAAACT----TACCAAAGAGACTGATGTAAATGCAT  -4
   CCTCTGCCCGCTGCAAGCTCTGGTAAAACTT---TACCAAAGAGACTGATGTAAATGCAT  -3
```

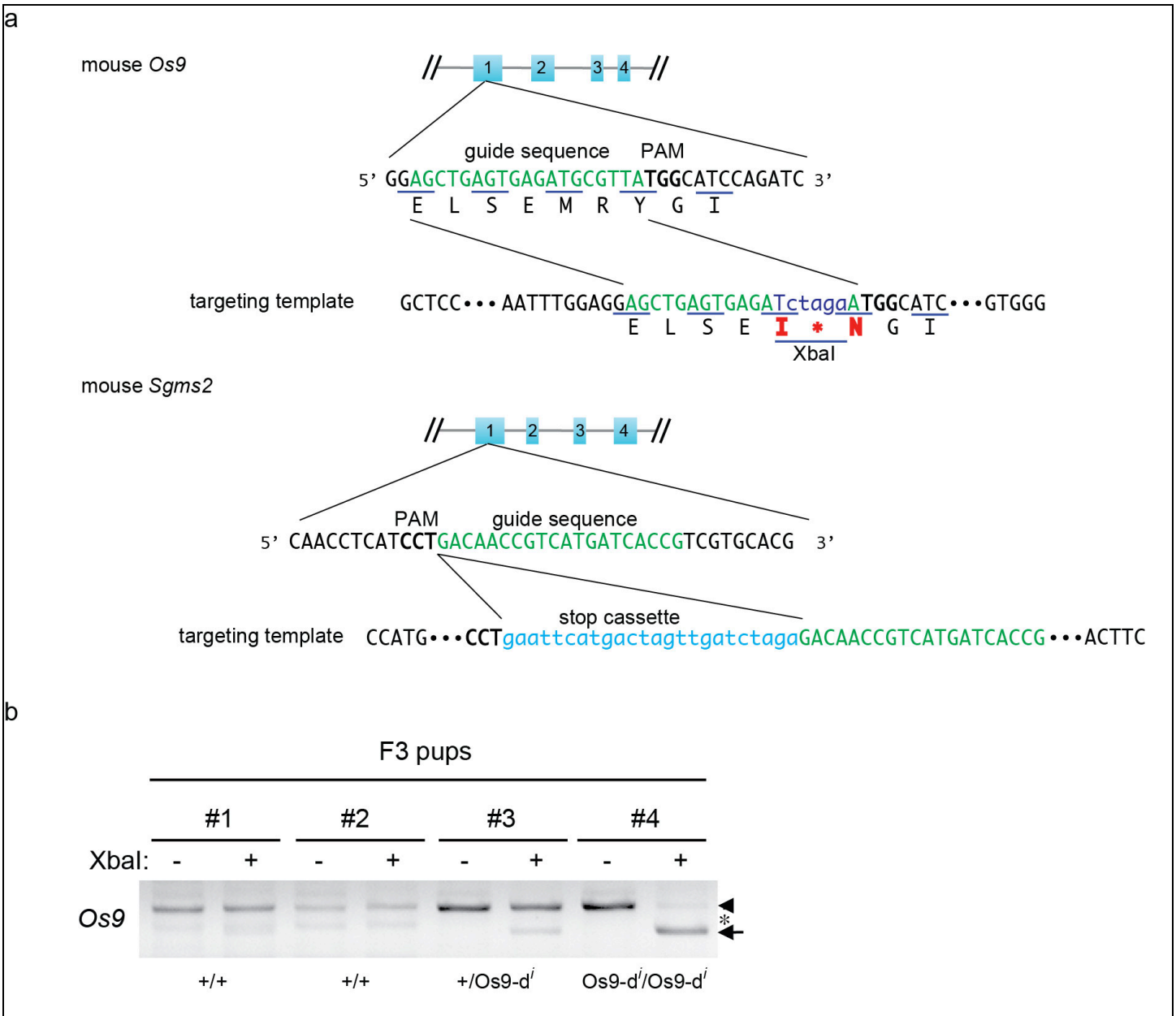
e



Supplementary Figure 5

Supplementary data for Fig. 2

(a) and (b). Fertilized zygotes were collected from oviducts of super-ovulated female mice. Zygotes were injected with 1 mM of Scr7 into the cytoplasm at the pronuclear stage. The injected zygotes were cultured up to the 16-cell stage. (a: the images of 2 cell stage, b: the images of 16 cell stage)
(c) The number of dead/live blastocysts was counted after injection. The total number of the zygotes injected is shown as the sum of blue and red column. The red column shows the number of zygotes that failed to develop, and the blue column represents the number of zygotes which developed to the blastocyst stage.
(d) The sequences of targeted alleles in deletion mutant (#2, #3, #9 and #11) shown in Fig. 2d.
(e) RFLP results of the *Igκ constant region*-targeted E10 embryos. The amplicon obtained using primer set #2 in figure 2e was digested with MboI, a recognition sequence present in the LPETG coding sequence. Digestion patterns of wildtype and inserted mutant amplicons are shown on the right side. The digested fragment specific for inclusion of the LPETG motif is shown as an arrow.



Supplementary Figure 6

Genotyping result of the F3 pups from the heterozygous parental F2 mice generated by Scr7-based approach

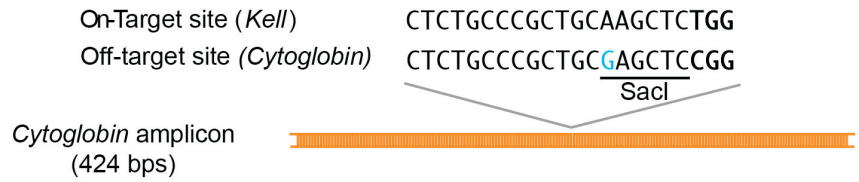
(a) Schematic of single guide (sg) RNA/ single-stranded oligos template-targeting site at the *Os9* exon 1 and the *Sgms2* exon 1. The guide sequence of the *Os9* sgRNA (Supplementary Table 1, *Os9* guide sequence is identical to the guide sequence used for the Os9 deletion #1 mice as in Supplementary Table 2) is in green. The protospacer-adjacent motif (PAM) is in bold. For stop codon-inserted mice at the Os9 exon1, the targeting single-stranded (ss) DNA template contained 66 and 55 nucleotide (nt)-homology arms on the left and right sides of the double strand breaks (DSBs), respectively. The original sequence, GCGTT, was converted to CTAGA, to introduce a stop codon and XbaI enzymatic site into exon 1 (*: stop codon). For Sgms2, a targeting template was designed to contain 88nt homology arms flanking the DSBs. The stop cassette, identical to that of TSG101 targeting template in Fig. 1, is inserted into the *Sgms2* exon 1. (b) The genotyping/RFLP result of Two-week-old pups from a parental pair of the heterozygous Os9 stop codon-inserted

F2 mice (C57BL/6 background) (The F2 mice are shown in Table 1). The direct sequencing results are shown at the bottom of a panel. The stop codon inserted on the *Os9* locus include unique XbaI enzymatic site. The PCR products were digested by XbaI. Arrow: digested bands, arrowhead: non-digested bands, *: non-specific bands. +: wild-type, Os9-d^{i}: stop codon-inserted mutant.

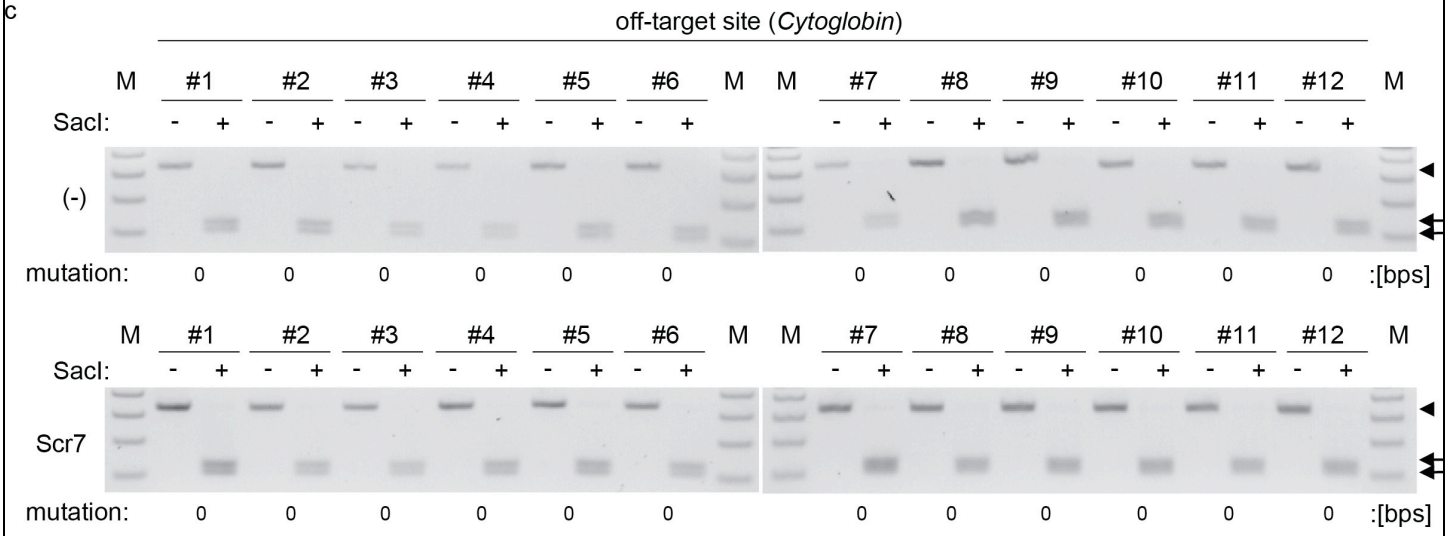
a

off target site sequence	score	mismatches	UCSC gene	locus	Gene
CCTCTGCCCGCTGCGAGCTCTGG	2.3	2MMs [5:15]	NM_030206	chr11:-116514995	Cytoglobin
GCTCTGCCCTCTGCCAGCTCAGG	1.6	2MMs [10:15]	NM_001114545	chr5:+118215619	Ksr2
TTACTGCCCACTGCAAGCTCTGG	1.3	4MMs [1:2:3:10]		chr13:+91000499	
CCCCCGCCCCCTGCAAGCTCCGG	1.3	4MMs [1:3:5:10]		chr8:-126285182	
GCGCTGTCTGCTGCAAGCTCAGG	1.0	3MMs [3:7:9]		chr8:-126285182	

b



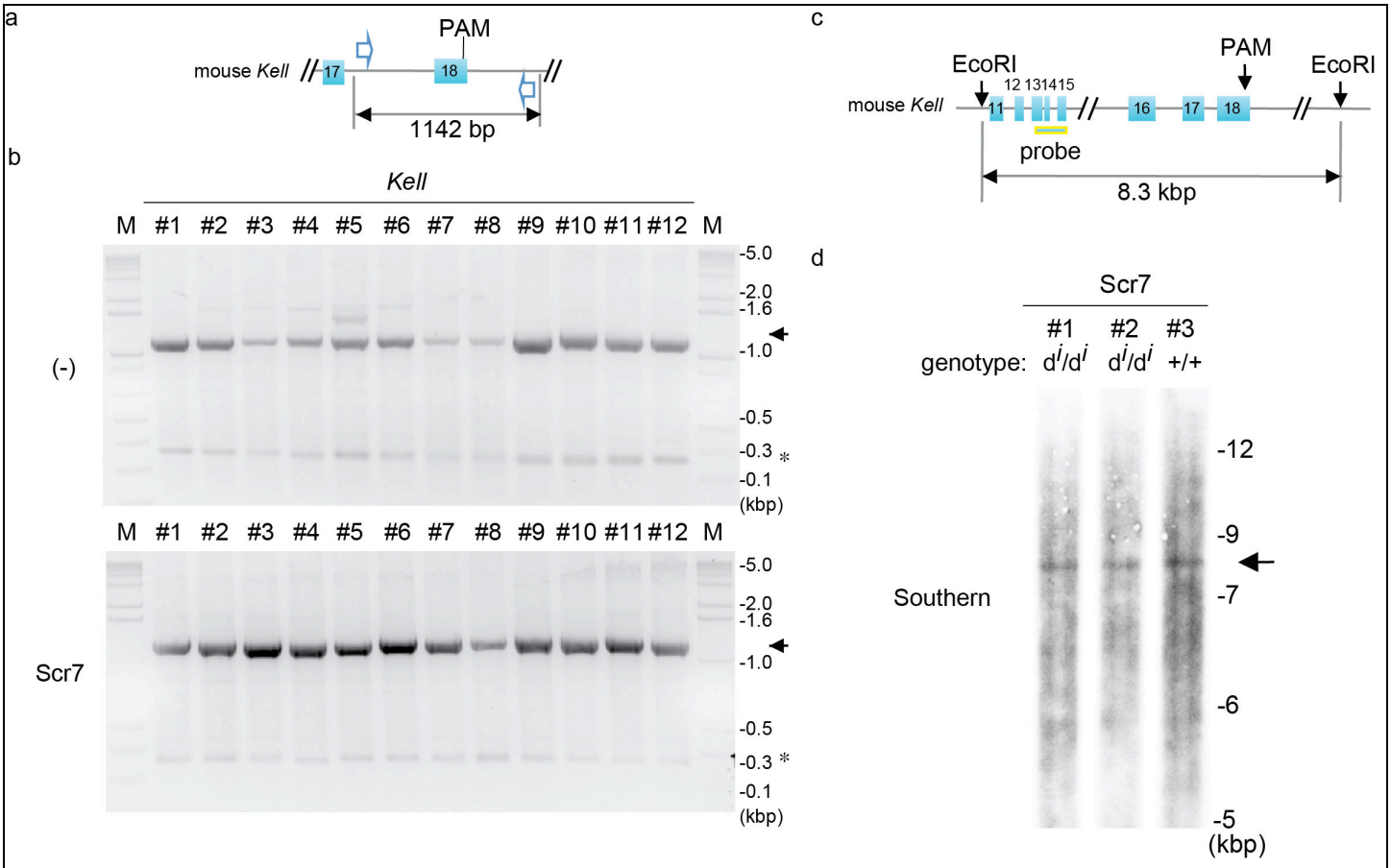
c



Supplementary Figure 7

Lack of off-target effects at a predicted site in the *Cytoglobin* locus

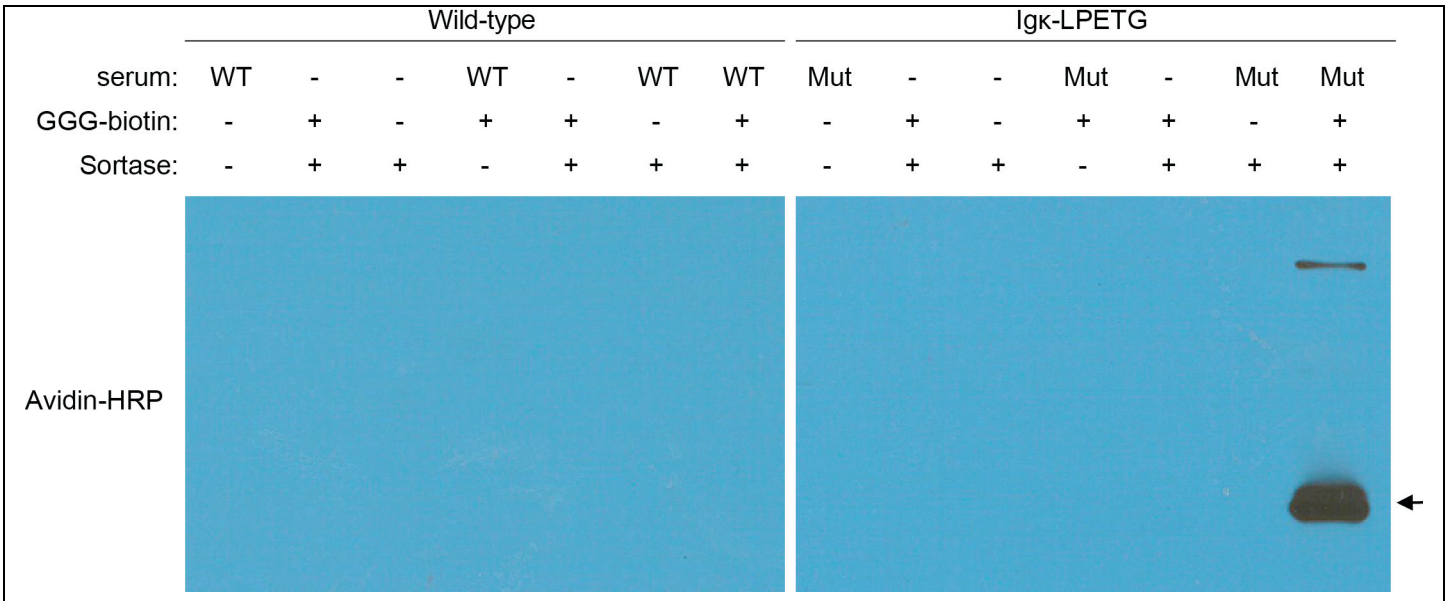
(a) Potential off-target sequences were predicted (Hsu et al., 2013 see Supplementary Methods). For searching off-target sites, 23 bp including PAM motif was utilized. The top 5 off-target sites predicted are shown. The actual mismatch on the *Cytoglobin* locus is one mismatch since the guide sequence and PAM motif used for mouse generation in this manuscript was 22 bps as shown in (b)
(c) A region flanking the most likely off-target site of the *Kell* guide sequence in the *Cytoglobin* locus was amplified by PCR using the same genome extracts as in Figure 1d. The amplicons of the *Cytoglobin* locus were digested by SacI enzyme to check for indels. Sequencing results of untreated amplicons (arrowheads) are shown at the bottom of each panel. (arrow: untreated amplicon, arrowhead: digested amplicon)



Supplementary Figure 8

Investigation of potential longer indels attributable to alternative NHEJ pathways

(a) A primer set was designed to check potential integration of longer indels at the *Kell* locus. A primer set that amplifies a wider region in the proximity of the DSB in the *Kell* locus was utilized (the primer sequences are shown in Supplementary Table 6). (The forward primer is 525 bp upstream from PAM motif, and the reverse primer is 612 bp downstream from PAM motif; the expected amplicon size is 1142 bp, as indicated by an arrow in b)
(b) The genotyping result using the *Kell* long primer. PCR products were loaded onto EtBr containing agarose gels. Arrow indicates the primer-specific amplicon. (arrow: target amplicon, *:nonspecific band) Numbers indicate genomic DNA from individual mice.
(c) Schematic overview of Southern blot to detect the *Kell* locus. The restriction enzyme EcoRI used for Southern blot analysis is shown, and the Southern blot probe is shown as yellow box.
(d) Southern blot analysis of Kell-LPETG-targeted allele. EcoRI-digested genomic DNAs of the E10 embryos, #1-3, treated by Scr7 were hybridized with 5' external probe. Expected fragment size: 8.3 kb. Arrow is the Kell-LPETG-targeted alleles.



Supplementary Figure 9

Biotin labeling of LPETG-modified immunoglobulin light chains (Ig) using sortase

Serum from a wild type mouse and a mouse expressing Igκ-LPXTG was collected and used in a sortase reaction as described in supplementary methods. The reaction mixture was adsorbed onto protein-G beads to remove non-Ig serum components and sortase. The beads were boiled in SDS sample buffer to release bound materials, which were then subjected to SDS-PAGE. Avidin-HRP was used to detect biotin-modified Ig light chains. The high MW polypeptide corresponds to incompletely dissociated Ig molecules.

mutation type	Gene	Background	Description	Newborn	mutant mouse		(mutant alleles)		NHEJ [%]	HDR [%]
					Deletion	Insertion	Deletion	Insertion		
deletion	OS9	BDF	Deletion sgRNA#1	7	2	-	4	-	28.6	-
			Deletion sgRNA #2	12	9	-	11	-	45.8	-
			Deletion sgRNA #3	5	1	-	2	-	20.0	-
	LRRC8D	BDF	Deletion	6	5	-	8	-	66.7	-
insertion	mesothelin	BDF	Stop cassette	18	7	2	7	4	19.4	11.1
	IgG3 constant region	BDF	LPXTG	9	7	2	8	3	44.4	16.7

Supplementary Table 1

Efficiencies of deletion and insertion mutagenesis in mice generated by a CRISPR approach without Scr7

Newborn pups were prepared using a conventional CRISPR method. The genotypes of the pups were analyzed by direct sequencing. Percentages of NHEJ and HDR were calculated by the relative number of the deletion or the insertion alleles over the total allele number.

Primer name	description	Sequence
pX330-R-Xba	sgRNA tail generic Rv	taagttatgtaacgggtac
mKell	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**CTCTGCCCGCTGCAAGCTC**gttttagagctaGAAAtagcaag
mIg κ constant region	T7+target sequence+head of sgRNA tail Fw3	TAATACGACTCACTATAGGG**GAGCTGGTGGTGGCGTCTC**gttttagagctaGAAAtagca
mOs9-1	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**AGCTGAGTGAGATGCGTTA***1
mOs9-2	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**CACTTCCAACGTGAAAGAG**gttttagagctaGAAAtagcaag
mOs9-3	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**GTGCTGCTGTCCAGTCTGT**gttttagagctaGAAAtagcaag
mMesothelin	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**CCAACAGCTCGACCCCTGC**gttttagagctaGAAAtagcaag
mIgG3	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**AGAACCTGTCTCGCTCCCC***1
mSgms2	T7+target sequence+head of sgRNA tail Fw	TAATACGACTCACTATAG**GGTGATCATGACGGTTGTC**gttttagagctaGAAAtagcaag

Supplementary Table 2

Primer sequences to produce template DNA amplicon for sgRNA synthesis

Minimal T7 promoter sequence is underlined, and guide sequence is in bold. *1: Vector-based transcription of sgRNA: The guide sequence-introduced pX330s were utilized for the sgRNA synthesis as described in Wang et al., 2013.

Target name	primer name	sequence
hTSG101	hTsg101 external Fw	AAGTGCTGTCACCTCAGGATATCTCC
	hTsg101extarnal Rv	AGACCAATCCTCAGTACTCTGTAGG
	hTsg101 stop internal Fw	CAGGCCGAGTTCATGACTAGTTGATCTAGA
	hTsg101 partial P5 FW	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCCTGTCTTTTCCTGTGAAGTGTTGTAGG
	hTsg101 partial P7 RV	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTCATCGATATCATTGTTTTCAGACTG
	MiSeq P5 universal FW	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	MiSeq P7 RV barcode #1	CAAGCAGAAGACGGCATACGAGAT**CCTGGTAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	MiSeq P7 RV barcode #2	CAAGCAGAAGACGGCATACGAGAT**TAAGCATG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
mTap1	mTAP1external Fw	TCAGCTCCACCAGCTCGAGCG
	mTAP1external Rv	TGCTGCCACATAACTGATAGCGAAGG
Venus	Venus internal Fw	ATGGTCCTGCTGGAGTTCGTGACC
	Venus internal Rv	GGACACGCTGAACTTGTGGCCGTT
mKell	Kell Geno Seq Fw	ATCTAACCCATCCCTATCACCCTATGG
	Kell Geno Seq Rv	TAATACCCAACGTTGTCCCCTAACCTC
	Kell longer Fw	ACACTACTCAGAGACCTCAGTCC
	Kell longer Rv	TTGCCAGTGTCCATGAAGCTTGC
	Kell southern probe Fw	TGGTGGGAGATCTCAAGAGG
	Kell southern probe Rv	ACCTGGAATGCAATGGCTAC
mIg κ constant region	IgKC Geno Fw	ATGCTGCACCAACTGTATCC
	IgKC Geno Rv	AAGACAGAGATCTCAAGTGC
LPETG	LPETG-specific Fw	ATCAGGAGGATCATTACCAGAGACAGGAGG
mOs9	OS9-1 Geno Seq Fw	GCAGAGACGGAGCGGAAGAT
	OS9-1 Geno Seq Rv	CAGTGGAAGAGAGAATAA
	OS9-2 Geno Seq Fw	GCTTATTCTCTCTTCCACTG
	OS9-2 Geno Seq Rv	ATCTAGTACACCAATGGCC
	OS9-3 Geno Seq Fw	CAAATGAGGATGCGTTACG
	OS9-3 Geno Seq Rv	AGATCTGGATGCCATAACG
mLRRC8D	LRRC8D-1 Geno Seq Fw	TTATGCTGATGGTAGCCATCTTTGCAGG
	LRRC8D-1 Geno Seq Rv	TTCACAGGCCGTCTCTGACAGCG
mMesothelin	Msln Geno Fw	CAGAGGTGCCCCACTGAC
	Msln Geno Rv	GCCTAGGGATCATTCCTGC
mIgG3	IgG3 Geno Fw	ACCTCTGTGTATGCTTCTAA
	IgG3 Geno Rv	GTTGTGGGGAAGAGTATGAA
Sgms2	Sgms2 Rv	AACGACTCCACCAACACTTACACAAGCC
	Sgms2 Rv	TAGGAATAACTAAATACGGGAGAAACTGG
TCR alpha	TCRalpha Geno Forward	TCCTGTGGCACCTTCCATTCCAAG
	TCRalpha Geno Reverse	GAGAACAGGACTTTTCCTGGGCACTCT

Supplementary Table 3

Primer sequences for genotyping, direct sequencing, deep sequencing and synthesis of Southern blot probe

For the deep sequencing primer, underlined sequences are P5 or p7 adapter sequence, and barcode sequences are bold.

Gene name	sgRNA target sequence
human TSG101	CTGTTCTGTTTTCAGGCCGAGG
mouse Tap1	TCCCGAGGTGCCGCTGCTCCGG
mouse Kell	CTCTGCCCGCTGCAAGCTCTGG
mouse Igκ constant region	GAGCTGGTGGTGGCGTCTCAGG
mouse Os9#1	AGCTGAGTGAGATGCGTTATGG
mouse Os9#2	CACTTCCAACGTGAAAGAGAGG
mouse Os9#3	GTGCTGCTGTCCAGTCTGTTGG
mouse LRRC8D	AAGGAGAAGAGAGACCCAACGG
mouse Mesothelin	CCAACAGCTCGACCCCTGCTGG
mouse IgG3	AGAACCTGTCTCGCTCCCCTGG
mouse Sgms2	GGTGATCATGACGGTTGTCAGG

Supplementary Table 4

Guide sequence (including PAM motif) of sgRNA for each gene targeting

Guide sequences of sgRNAs used are shown. The PAM motif is underlined. From the PAM motif, 19 bps were utilized for targeting each gene.

Inserted block	*TTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATT AGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTA CCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG***G***tctagagaattcactagt*GTTTTAGA GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT

Supplementary Table 5

A template vector sequence for site-directed mutagenesis

The U6 promoter is in italics (a bold G at the end of italicized sequence is a starting position of transcript), control template sequence, containing XbaI, EcoRI and SpeI restriction enzyme sites, is underlined, and the sgRNA tail is capitalized. The sequences were inserted into pCDH-hygro vector as described in the Methods section.

primer name	primer sequence
hTSG101 sense	GGAAAGGACGAAACACCG**CTGTTCTGTTTTCAGGCCG**GTTTTAGAGCTAGAAATAGC
hTSG101 antisense	GCTATTTCTAGCTCTAAAAC**CGGCCTGAAAACAGAACAG**CGGTGTTTCGTCCTTTCC
mTap1 sense	GGAAAGGACGAAACACCG**TCCCGAGGTGCCGCTGCTC**GTTTTAGAGCTAGAAATAGC
mTap1 antisense	GCTATTTCTAGCTCTAAAAC**GAGCAGCGGCACCTCGGGA**CGGTGTTTCGTCCTTTCC

Supplementary Table 6

Primers for site-directed mutagenesis cloning

The guide sequence of sgRNA is in bold.

Gene name	Description of insertion type	template type	Length of homologous region	template sequence
hTsg101	insertion of a stop codon cassette:	double stranded DNA	left: 100 bp right: 100 bp	**GTTGTTAGCCTGTCTTTTCCTGTGAAGTGTTGTAGGTGTCTGACAAGTGGATAATTATGTACAGTTATTAATGCTTGGA CTGTTCTGTTTTCAGGCCGAG**TTCATGACTAGTTGATCTAGA**GTTGATAAAAACATAGAACTTTTGAAAAAGAAGGATG AAGAACTCAGTTCTGCTCTGGAAAAAATGGAAAATCAGTCTGAAAACAATGATATCGATGAAG***1
mTap1	for insertion of a Venus reporter gene:	double stranded DNA	left: 213 bp right: 213 bp	**ACGCGCACGCCCTCGGACCCGCCCTTCTTCCTTCCCCACGGAGACTCCTGTGCAGCGCGGACGTCGAGAGTCCCAGGCT AGGACCAGACTCTGGACAGCTCACGCTCGATGGCTGCGCACGTCTGGCTGGCGGCCGCCCTGCTCCTTCTGGTGGACTG GCTGCTGCTGCGGCCCATGCTCCCGGGAATCTTCTCCCTGTTGGTTCCCGAGGTG**gaattcGACTATAAGGACCACGAC GGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTG TGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTT CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAAGCTG CCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGC AGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTA CAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAG GACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGA ACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAA CACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCAAGCTGAGCAAAGACCCC AACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA AGTAA**tctaga**CGGGTCTGGGTGGTGGGCCTGAGTCGCTGGGCCATCCTAGGACTAGGGGTCCGCGGGGTCCTCGGGGT CACCGCAGGAGCCCATGGCTGGCTGGCTGCTTTGCAGCCGCTGGTGGCCGCACTGAGTTTGGCCCTGCCTGGACTTGCC TTGTTCCGAGAGCTGGCCGCCTGGGGAACACTCCGGGAGGGTGACAGCGCTGGATTACTGTACTGG***2**
	positive control for PCR analysis	double stranded DNA	left: 914 bp right: 660	**CTCCACAGACCCCTGGACACCACACTTCTGAGCAGGGATACCCTACGTTCACTGTGGAGCGCAAGCGCTGGGCGCTACA AGCTTCCTAAGTCTCTTGGTTTACTTCCAGAGCTTTAGGGGTCCCTGATCACTCCACCCGCTGACTCTCACCCCGAGAC CCATTACCCCGGTGTGGACTTCTTCCGTCCGGAACGAGCCGGCGGTAGGTGCTCCTGCCCGCAGCATCTCAGCAGAGCG GGGCTCGGCTTTCCAATCAGCGGCTGCGCGCGGTGCAGGCAACTTGCAGACTGAGGCCCCGCCCCATCATCGCGCAAGG GGCGTGCCGTTCTACCAGCATTTGGCGCCCAGAGCAAACCTGAGCAGGGCAAATCTGCCCAGAGACAGGTGACGACAGA GGGTCCTGCCCTCAATCTGGGGTGGGGCCTGGGATGGGAAAATTCACGCAAGCAAGTTAAGGGGGCTGGGGAAGAAGAG GAGAATGAGATTCATGGAGAAGAACACGACAGGCCAGGGCTGCTAGGCAGAACTCCAACTACAGCTTCAGCGGCAGCTT CCAGAACAGCCTGAGCAAGCCAGTCTCAGAAGGAGGCGTGTCTAGTGATTCGAGGTCGGCTTTCGGTTTCTTCTTCCTC TAAACGCCAGCACTTCTAGTCAGCTCCACCAGCTCGAGCGGGTTCCCGGGACTTTACGCGCACGCCCTCGGACCCGCCC TTCTTCCTTCCCCACGGAGACTCCTGTGCAGCGCGGACGTCGAGAGTCCCAGGCTAGGACCAGACTCTGGACAGCTCAC GCTCGATGGCTGCGCACGTCTGGCTGGCGGCCGCCCTGCTCCTTCTGGTGGACTGGCTGCTGCTGCGGCCCATGCTCCC GGGAATCTTCTCCCTGTTGGTTCCCGAGGTG**gaattcGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATT GATTACAAAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTGTGAGCAAGGGCGAGGAGCTGTTCA CCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGG CGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG ACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCA TGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAG CTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGA TCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGT GCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA**tctaga**CGGGTCTGGGTGG TGGGCCTGAGTCGCTGGGCCATCCTAGGACTAGGGGTCCGCGGGGTCCTCGGGGTCACCGCAGGAGCCCATGGCTGGCT GGCTGCTTTGCAGCCGCTGGTGGCCGCACTGAGTTTGGCCCTGCCTGGACTTGCCTTGTTCCGAGAGCTGGCCGCCTGG GGAACACTCCGGGAGGGTGACAGCGCTGGATTACTGTACTGGAACAGTCGTCCAGATGCCTTCGCTATCAGTTATGTGG CAGCATTGCCCGCAGCCGCCCTGTGGCACAAGTTGGGGAGCCTCTGGGCGCCCAGCGGCAACAGGGACGCTGGAGACAT GCTGTGTCGGATGCTGGGCTTCCTGGGCCCTAAGAAGAGACGTCTCTACCTGGTTCTGGTTCTCTTGATTCTCTCTTGC CTTGGTAAGGGAGACTGGGTGAAAGAGCAGGCCACAGGGGACAGGGCAATGGGCATTCCAGGCAGAAAGGGAGAGTAAA GTAGTCCCGGGAAGCGTCAGAGACACTAAGGAGGCAACTTAGGCGGAAAGAGAAGGACCCAGGCCCGAGAAGGGGTCCA GACAAGGGCGTTGGGGACAGGGTCTTCTGGGAGCAGAACTTCACATCATAGCCCAAATGTGAAGGTTTCTTTCCACACC GCCATCTTCTCCTCT***2
mKell	Insertion of LPETG	oligo DNA	left: 80 nt right: 80 nt	**TGAGCAATACTCCAGATTTTGCCAAACATTTTCATTGTCCACGTGGGACCCTTCTGAATCCCTCTGCCCGCTGCAAGCT C**GGAGGATCAGGAGGATCATTACCAGAGACAGGAGGA**TGGTAAAACTTGGCTACCAAAGAGACTGATGTAAATGCATGG GCTGCTTGTGAGTCCATCCTTGAAGTCAAAATAAATCT***3
mIgκ constant region-LPETG	Insertion of LPETG	oligo DNA	left: 79 nt right: 64 nt (from deletion)	**ACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGT** GGAGGATCAGGAGGATCATTACCAGAGACAGGAGGA**TGAAGACAAAGGT^GAGACGCCACCACCAGCTCCCCAGCTCCA TCCTATCTTCCCTTCTAAGGTCTTGGAGGCTTCC***3
mMesothelin:	Stop codon and XbaI enzymatic site	oligo DNA	left 65 nt right 56 nt	**GGTGGGACAAGTGGGGACCTCAGAGTCATTGTTATCCACAGACCATGGCCTTGCCAACAGCTCGA**CTCTA **GATGGGGTCCTGTGGAAGTCCCATCTGCAGCCGAAGCTTCCTACTGCTTCTCCTTA***4
mIgG3	LPETG reconstitution based on original sequence	oligo DNA	left: 61 nt right: 54 nt	**AATTTTTACCTGCTCCGTGGTGCATGAGGCTCTCCATAACCACCACACACAGAAGAACCTG**c**CTCGC**a**CC**gg**TGGTAAA TGAGAACAGCACCTAGCCATTCCTCGGGTCTTACAAGACACTGATAC***5
Os9	insertion of a stop codon cassette:	oligo DNA	left: 66 nt right: 55 nt	***gctcctgttacctgcgcgtctgacgggcggtgtcgggagcctgaatttggaggagctgagtgagat***CTAGA***atggcatc cagatcttgccgttgcctgtcatgggagggcaggtaaggggtgtggg****4
Sgms2	insertion of a stop codon cassette:	oligo DNA	left: 88 nt right: 88 nt	**CCATGCCCAACGACTCCAAGAACAAGTTTCCCCTGGAGTGGTGGAAAACAGGCATCGCCTTTGTGTATGCGCTCTTCAA CCTCATCCT**gaattcatgactagttgatctaga**GACAACCGTCATGATCACCGTCGTGCACGAGAGGGTCCCTCCCAAG GAGCTCAGCCCTCCGCTCCCAGACAAGTTTTTTGATTACTTC***1

Supplementary Table 7

Sequence of double-stranded donor and single-stranded targeting oligo template for all genes shown here, homology arms are shown in bold typeface.

*1: The stop cassette is underlined. *2: The Venus sequence is underlined. *3: The LPETG+linker encoding sequence is underlined and ^ is deleted bases. *4: The reconstituting sequence used to change the original sequence to XbaI + stop is underlined. *5: The reconstituting sequence used to change original sequence to LPETG is underlined.

Supplementary Text

HDR-mediated targeting

To control the timing of Cas9-mediated DSBs, we used cell lines encoding a genomically integrated, doxycycline-inducible Cas9 cassette. Next, we transduced these cell lines using a lentiviral system to introduce a second construct that stably expresses sgRNAs targeting a specific gene of interest (Supplementary Fig. 1a, stable cell preparation). We then confirmed reduced levels of TSG101 protein after Cas9 induction (Supplementary Fig. 1b).

To enable template-based HDR, these cells were transfected with an additional plasmid donor encoding the intended insert flanked by 5'- and 3'-end homology arms, and Cas9 was induced by the addition of doxycycline (Fig. 1a, stimulation of HDR).

Cell viability upon Scr7 treatment

To assess potential toxicity of Scr7, we checked cell viability following exposure to different concentrations of Scr7 (Supplementary Figs. 2a-c). Cas9-expressing sgRNA stable cells were transfected with the plasmid donor and treated with Scr7 and doxycycline. Cell viabilities were then checked by MTT assay. A549 cells were slightly more susceptible to toxic effects of Scr7 than MelJuSo cells (Supplementary Figs. 2b-c), and an increased concentration (10 μM) of Scr7 had a more toxic effect on cell viabilities of both cell lines. At concentrations less than 10 μM, cells recovered after 3 days of Scr7-treatment. To maintain healthy cells capable of entering S/G2, which is necessary for HDR, we chose a range from 0 to 1 μM of Scr7.

Germline transmission

In the preceding experiments, mice were harvested at E10. The embryos appeared phenotypically normal, but we wanted to confirm that Scr7 does not adversely affect the health of older mice. We obtained live mice containing either stop codon-introduced *Os9* or stop cassette-inserted *sphingomyelin synthase 2*[1] mutations (Supplementary Fig. 5a and Table 1), which were generated by the Scr7-based approach. For both mutant lines, the adult mice appear healthy (up to at least 4 months of age), and germline transmission occurred successfully, as we obtained pups carrying the stop-codon at the targeted site of the *Os9* locus from the heterozygous parental mice (Supplementary Fig. 5b).

Effect on off-target by Scr7 treatment

Since off-target effects can occur in CRISPR-based generation of mutant mice[5], we checked for off-target effects at the most likely sites for the *Kell*-targeting guide sequence. We determined that the sequence most similar to the *Kell*-targeting guide occurs in the *Cytoglobin* locus, which has 1 mismatch relative to the *Kell* guide sequence (Supplementary Fig. 7a). We observed no off-target effects at the *Cytoglobin* locus in either Scr7-treated or untreated embryos (Supplementary Fig. 7b), consistent with a previous report[5].

Long indel analysis

A deficiency of *Ligase IV*/*XRCC4* induces A-NHEJ pathways, which can potentially introduce longer indels at a low frequency[25,26]. We thus checked whether the *Kell* locus proximal to the DSB contained any longer indels (Supplementary Fig. 8). Since the length of deletion patterns in *Xrcc4* knockout cells was reported to be less than 1kbp[26], we used a different primer set that can amplify a larger region (1142 bp) of the *Kell* locus to detect the possible occurrence of longer indels (Supplementary Fig. 8). We did not detect any longer indels, although indels larger than ~1.1 kb would not have been detected by our analysis. We also only observed homozygous mutant mice. This may be an artifact of the analysis, since one allele is often undetectable by PCR in genome-edited mice[4,5]. To confirm whether the homozygous mutants analyzed in Fig. 2d carried such hidden alleles, we performed Southern blot analysis (Supplementary Figs. 8c and d). We observed the expected ~ 8 kbp band corresponding to the *Kell* locus (arrow) upon digestion with EcoRI in the genomic DNA of mutant embryos (#1 and #2)--which were observed as homozygous mutant in Fig. 2d. These bands are similar intensities to those of the wild-type embryo (#3), suggesting that these embryos do not lose a copy of the entire *Kell* gene by deletion.

Supplementary references

1. Hailemariam, T.K. et al. Sphingomyelin synthase 2 deficiency attenuates NFkappaB activation. *Arteriosclerosis, thrombosis, and vascular biology* 28, 1519-1526 (2008).